REMARKS

This paper is in response to the official action dated February 16, 2006. This paper is timely-filed as it is accompanied by a petition for an extension of time to file in the third month and a check covering the requisite fee of $1020.00.

Claims 1-49 are pending, but claims 6, 8, 9, 18, 19, 22-25, 30-32, 36-47, and 49 have been withdrawn from further consideration. Claims 1-5, 7, 10-17, 20, 21, 26-29, 33-35, and 48 are presently at issue.

Claims 35 and 48 remain rejected under 35 U.S.C. §112, first paragraph, as assertedly failing to comply with the written description requirement. Additionally, claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48 have been rejected under 35 U.S.C. §112, first paragraph, as assertedly failing to comply with the written description requirement.

Also, claims 1, 2, 4, 5, 7, 10, 11, 13-17, 20, 21, and 28 have been rejected under 35 U.S.C. §102(b) as assertedly anticipated by Hamilton, *et al.*, *J. Med. Chem.*, *38*:1650-1656 (1995) ("Hamilton") in light of Lee, *et al.*, *Structure*, *3*:1333-1340 (1995) ("Lee").

The various bases for the claim rejections are addressed below in the order presented in the action. Reconsideration of the application is respectfully requested.

CLAIM REJECTIONS – 35 U.S.C. §112, FIRST PARAGRAPH

Claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48 have been rejected under 35 U.S.C. §112, first paragraph, as assertedly failing to comply with the written description requirement. Applicant respectfully traverses the 35 U.S.C. §112, first paragraph, rejections of claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48.

Statutory law requires that the specification shall contain a written description of the invention. *See* 35 U.S.C. § 112, first paragraph. The courts have interpreted that provision as requiring that the description of the invention be sufficient to allow one of skill in the art to recognize that Applicant was in possession of the subject matter claimed. Vas-Cath v. Mahurkar, 935 F.2d 1555 (Fed. Cir. 1991); *accord*, M.P.E.P. §2163 (I). Possession is shown by describing the claimed invention with all of its limitations using descriptive means such as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. Possession may be shown by describing an actual reduction to practice or by describing distinguishing identifying characteristics sufficient to show that Applicant was in possession of the claimed invention. *See, e.g.*, M.P.E.P. § 2163 (I).

Claims 35 and 48

Claims 35 and 48 have been rejected as failing to comply with the written description requirement because they recite "ftsz." However, Applicant's possession of methods directed to the genus of molecules comprising an α/β domain structure, in combination with the teachings regarding the Rossmann fold structure of ftsz and allosteric effector small molecules, provides sufficient written description of the subject matter recited in claims 35 and 48.

At page 4 of the action, the examiner acknowledged that the application provides an adequate written description of "methods of modulation of generic proteins having alpha/beta domains...," but asserted that the "description of a method of using a genus does not equate to description of every individually claimed member of the genus...." While recognizing that a genus may not support a species in certain situations, the Applicant respectfully submits that the examiner's position is legally flawed in view of the present facts where (1) there is relevant description in the application as originally-filed describing the structure and identity of the species at issue and (2) possession of the genus has been found, as explained in more detail below.

There is relevant disclosure in the application describing the Rossmann fold structure of ftsz despite the examiner's assertion that such disclosure is lacking at page 3 of the official action. For example, when discussing the demonstrated atomic structure of ftsz at pages 19 and 142, the application references Nogales *et al.*, Nature Structural Biology, 5:451-458 (1998), which itself discloses that the nucleotide binding domain (or Rossmann fold structure - *see* application at page 1, lines 25-26) of ftsz includes a central β sheet structure comprising six β sheet strands positioned in a 321456 orientation. Such information, when considered in further view of the application teachings that "[a] Rossmann fold structure in a protein comprises a beta sheet structure wherein individual beta sheet domains of the protein are positioned in either parallel, antiparallel, or mixed orientations" and the "Rossmann fold structure in said first molecule comprises a β sheet having β sheet strands positioned in a 321456 or 231456 orientation (*see* application at pages 5 and 9, respectively), constitutes strong evidence corroborating Applicant's possession of the Rossmann fold structure of ftsz at the application filing date. Moreover, claim 48 is an *original claim* indirectly dependent from claim 16, which recites structural

details regarding the Rossmann fold structure of ftsz (i.e., the Rossmann fold in ftsz comprises a β sheet having β sheet strands positioned in a 321456 orientation).

Additionally, applicant's description of methods directed to the genus of molecules comprising an α/β domain structure is applicable to each of the species disclosed in the application – because, as discussed in Applicant's previous response, each of the species of the genus shares common structural characteristics. Thus, with respect to the examiner's assertion that the specification does not describe "effectors of any type of FtsZ" at page 3 of the action, the disclosure (throughout the application) pertaining to the small molecule allosteric effector compounds is applicable to the methods of claims 35 and 48. Accordingly, the disclosure that the claimed methods use allosteric effector molecules such as diaryl compounds, more preferably diaryl sulfide compounds and diarylamide compounds (*see* application at page 15, lines 2-9) and the numerous representative diaryl compounds illustrated in Table 2 constitute disclosure of allosteric effector small molecules of ftsZ, and further support the Applicant's possession of claims 35 and 48 at the time the application was filed.

Similarly, with respect to the examiner's assertion that the specification does not describe a "method utilizing FtsZ that comprises effectors, or the claimed structural limitations of FtsZ such as Rossmann folds, or the structural limitations of an FtsZ effector such as small molecule, diaryl compounds..." at page 3 of the action, the description of the methods of modulating binding interaction between a first molecule comprising an α/β domain structure and a binding partner molecule, found variously throughout the application, is applicable to the methods of claims 35 and 48. The description of the methods directed to the genus of molecules comprising an α/β domain structure, when considered in view of the application disclosure of (1) the Rossmann fold structure of ftsz and (2) allosteric effector small molecules of ftsz, establishes Applicant's possession of the subject matter of claims 35 and 48 at the time the application was filed. Furthermore, at least page 8, lines 20-21 and Example 20 of the specification specifically disclose methods of modulating binding interaction between ftsz and GTP in contrast to the examiner's assertion that such disclosure is lacking.

To the extent the examiner is relying on University of Rochester v. G.D. Searle & Co., 358 F.3d 916 (Fed. Cir. 2004), Applicant respectfully submits that Rochester is inapposite with respect to the presently claimed subject matter. Rochester involved an extreme fact situation where the patentee claimed a method of administering (any) COX-

2 inhibitor, but failed to disclose a *single* example of such an inhibitor. Moreover, the Court noted that "there is no language here, generalized or otherwise, that describes compounds that achieve the claimed effect." This situation is not present here where Applicant specifically disclosed numerous representative allosteric effector small molecules and further disclosed their applicability to the claimed methods.

For all of the foregoing reasons, Applicant submits that the subject matter of claims 35 and 48 is described such that one of skill in the art would recognize that Applicant possessed that subject matter at the time of filing. Accordingly, the rejection of claims 35 and 48 as lacking written description has been overcome and should be withdrawn.

Claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48

Claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48 have been rejected as failing to comply with the written description requirement because they recite "diarylamide compounds." Applicant respectfully traverses the rejections.

At page 4 of the action, the examiner asserted that amides are compounds with an acyl group linked to an NH_2 group and that the application does not show such a structure. The examiner, however, is only partially correct. Amides may, but need not, comprise an acyl group linked to an NH_2 group; more accurately, an amide comprises an acyl group linked to NR_1R_2 where either or both of R_1 and R_2 may be hydrogen.

The application discloses numerous diaryl molecules in Table 2 and at least Compounds A, B, D, F, G, H, I, K, L, M, P, Q, T, U, V, W, AQ, AR, AS, and AAC further comprise an amide group, and thus, in view of the application disclosure, would be classified as diarylamide compounds by one of ordinary skill in the art. Furthermore, several of these compounds have been demonstrated in the claimed methods. For example, see the description relating to compounds AQ, AR, and AS in Example 4, and relating to compounds D, K, and W in Example 9. Therefore, the examiner's contention that the "specification does not show a structure or describe a working example of a diarylamide effector that regulates binding as part of the claimed method" is incorrect.

In view of the above discussion, Applicant submits that the subject matter of claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48 is described such that one of skill in the art would recognize that Applicant possessed that subject matter at the time of filing.

Accordingly, the rejection of claims 3-5, 7, 12-17, 20, 21, 26-29, 33-35, and 48 as lacking written description has been overcome and should be withdrawn.

CLAIM REJECTIONS -- 35 U.S.C. §102

Claims 1, 2, 4, 5, 7, 10, 11, 13-17, 20, 21, and 28 have been rejected under 35 U.S.C. §102(b) as assertedly anticipated by Hamilton *et al.* in light of Lee *et al.*

It is well-established that each and every limitation of a claimed invention must be present in a single prior art reference in order for anticipation to occur. *See*, for example, *C.R. Bard, Inc. v. M3 Systems, Inc.*, 157 F.3d 1340, 1349 (Fed. Cir. 1998). The standard for anticipation is one of strict identity. This standard has not been satisfied with respect to claims 1, 2, 4, 5, 7, 10, 11, 13-17, 20, 21, and 28.

Hamilton discloses compounds that inhibit neutrophil recruitment into inflamed tissue by interrupting the interaction between leukocyte integrin molecules and corresponding ligands on the endothelium. Hamilton specifically discloses inhibiting neutrophil adherence under conditions in which adherence is mediated by Mac-1 and LFA-1. Hamilton, however, does not disclose contacting a first molecule comprising an α/β domain structure, said α/β structure comprising an allosteric regulatory site, with an allosteric effector small molecule that *interacts with said allosteric regulatory site* and promotes a conformation in a ligand binding domain of said α/β structure that modulates binding between said first molecule and said binding partner molecule. Although Hamilton states that the compounds do "not appear to act by directly blocking ICAM-1/Mac-1 interactions, Hamilton ambiguously teaches that "[m]echanistic studies to date are consistent with a mechanism involving inhibition of signal pathways in leukocytes." *See* Hamilton at page 1654. Hamilton therefore does not imply or suggest contacting a first molecule comprising an α/β domain structure, said α/β structure comprising an allosteric regulatory site, with an allosteric effector small molecule that *interacts with said allosteric regulatory site*. Lee does not address this deficiency.

Because Hamilton does not disclose methods where the binding interaction between a first molecule and a binding partner molecule is modulated by contacting an allosteric regulatory site of the first molecule with an allosteric effector small molecule, the examiner is relying on the doctrine of inherency. However, "[t]o establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency ... may not be established by probabilities or

possibilities." *In re Robertson*, 169 F.3d 743, 745 (Fed. Cir. 1999); *see also* M.P.E.P. §2112.

Here, there is no reason to infer that the missing descriptive matter is necessarily present in Hamilton. In fact, Hamilton references a (then-submitted) journal article by Endemann, *et al.* (copy attached hereto as Attachment A),[1] which discloses that leumedins acted on A23187-activated leukocytes after a binding partner was introduced. *See* Endemann at page 11. This observation constitutes strong evidence that the missing descriptive matter is not necessarily present in Hamilton.

Because Hamilton does not make clear that the binding interaction between the leukocytes and its binding partner molecule (present in the serum-coated wells or on the endothelial cells) is modulated by contacting an allosteric regulatory site on the leukocytes with an allosteric effector small molecule, the examiner may not rely on the doctrine of inherency in order to establish same.

For the reasons set forth above, it is respectfully submitted that the outstanding anticipation rejections of claims 1, 2, 4, 5, 7, 10, 11, 13-17, 20, 21, and 28 have been overcome and therefore should be withdrawn.

CONCLUSION

It is submitted that the application is in condition for allowance. Should the examiner wish to discuss any matter of form or procedure in an effort to advance this application to allowance, he is respectfully invited to telephone the undersigned attorney at the indicated telephone number.

Respectfully submitted,

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August 16, 2006

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[1] The article eventually published as Endemann, *et al.*, *J. Pharmacol. Exp. Ther.*, *276(1)*:5 (1995).

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Novel Anti-Inflammatory Compounds Prevent CD11b/CD18, $\alpha_M\beta_2$ (Mac-1)-Dependent Neutrophil Adhesion Without Blocking Activation-Induced Changes in Mac-1

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ABSTRACT

Leumedins are small organic molecules with anti-inflammatory properties *in vivo*. We report here that leumedins inhibit the CD11b/CD18 $\alpha_M\beta_2$ (Mac-1)-dependent adherence of neutrophils to serum proteins. The activation of neutrophils leading to adherence *via* Mac-1 is associated with an increase in cell surface Mac-1 level, and with an increased affinity of Mac-1 for adhesion partners. Inhibition of neutrophil adherence by leumedins does not require blocking the recruitment of Mac-1 from intracellular granules to the cell surface. Furthermore, leumedins do not block the expression on Mac-1 of the epitope for an "activation-specific" antibody (CBRM1/5). Time course studies show that leumedins inhibit adherence by targeting an event which occurs concurrently with changes in Mac-1 level and induction of the CBRM1/5 epitope. Therefore, leumedins block an unknown process which is permissive for Mac-1-dependent adherence.

Movement of neutrophils from blood vessels into tissues is an appropriate physiological response to infection or injury. When this process is blocked, as in the human congenital disease Leukocyte adhesion deficiency, the body is unable to adequately fight infection. Leukocyte adhesion deficiency-I results from a deficiency in cell surface expression of the β_2 integrin (CD18) subunit, thus implicating the β_2 integrins LFA-1 (CD11a/CD18), Mac-1 and CD11c/CD18 in neutrophil extravasation (Kishimoto *et al.*, 1987). These molecules have been shown to mediate the firm adhesion of neutrophils, T lymphocytes and monocytes to endothelial cells (Mentzer *et al.*, 1986; Arnaout *et al.*, 1988). However there is evidence that excessive mobilization of neutrophils can contribute to tissue damage through release of proteinases and reactive oxygen species (Weiss, 1989). Antibodies to CD18 can attenuate tissue damage in models of inflammation (Harlan *et al.*, 1992), and anti-inflammatory agents which work by blocking β_2 integrin function have been proposed.

Both Mac-1 and LFA-1 are important in the adherence of neutrophils to endothelial cells. LFA-1 binds to ICAM-1 and ICAM-2 on endothelial cells (Marlin and Springer, 1987; Staunton *et al.*, 1989), whereas known adhesion partners for Mac-1 are ICAM-1 (Diamond *et al.*, 1990) and several non-endothelial proteins including fibrinogen (Altieri *et al.*, 1988), iC3b (Arnaout *et al.*, 1983) and Factor X (Altieri and Edgington, 1988). On resting neutrophils, the β_2 integrins are not able to mediate adherence to protein substrates or to endothelial cells. Through a poorly understood series of events, activation of neutrophils with TNF-α, PAF, fMLP and other factors allows them to adhere to endothelial cells in a Mac-1- and LFA-1-dependent manner (Diamond and Springer, 1994). One consequence of neutrophil activation is a 10-fold increase in the cell surface level of Mac-1 due to the recruitment of Mac-1 from intracellular granules to the plasma membrane. Studies have shown that this increase in Mac-1 level is not necessary for neutrophil adherence *in vitro*. Incubation with an anion transport inhibitor, or at 16°C, can block the increase in Mac-1 level without blocking CD18-dependent adherence to endothelial cells (Vedder and Harlan, 1988; Schleiffenbaum *et al.*, 1989).

Evidence has accumulated that, after activation of leukocytes, LFA-1 and Mac-1 undergo structural changes concurrent with increasing their affinities for adhesion partners (Lollo *et al.*, 1993; Diamond and Springer, 1994). Monoclonal antibodies, NKI-L16 and 24, have been described which bind CD11a on activated, but not resting, T lymphocytes (van

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ABBREVIATIONS: Mac-1, CD11b/CD18, $\alpha_M\beta_2$; LFA-1, CD11a/CD18, $\alpha_L\beta_2$; ICAM-1, intercellular adhesion molecule 1, CD54; TNF-α, tumor necrosis factor-α; PAF, platelet activating factor, L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl; fMLP, N-formyl-Met-Leu-Phe; PMN, polymorphonuclear leukocytes; RBC, red blood cells; HBSS, Hank's buffered salt solution with 0.2% glucose, 0.9 mM magnesium chloride, 1.26 mM calcium chloride and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2; PBS, phosphate-buffered saline; FBS, fetal bovine serum; IgG, immunoglobulin G; FACS, fluorescence-activated cell sorter; C5a, complement 5 activation fragment.

Kooyk *et al.*, 1991; Cabanas and Hogg, 1993). Similarly, activation of monocytic cells leads to the formation of an epitope on Mac-1 recognized by the antibody 7A10 (Elemer and Edgington, 1994). A second antibody (CBRM1/5) has been described which binds Mac-1 on monocytes and neutrophils after cell activation. The CBRM1/5 epitope is found only on a subpopulation (10–50%) of Mac-1 molecules on activated cells, but the antibody is able to block Mac-1-dependent adhesion (Diamond and Springer, 1993). Increased adhesion could be due to conformational changes in individual integrin molecules, causing a change in affinity for ligands. Alternatively, it could be due to clustering of integrin molecules at adhesion sites, leading to an increased number of interactions with ligand, and resulting in an overall increase in avidity of attachment. It is not known whether recruitment of integrins is sufficient to cause neutrophil adherence, or whether other parallel processes are necessary. Antibodies have been described which bind to β_2 and α_L subunits on resting cells and induce adherence, suggesting that structural changes in the integrins alone are sufficient (Robinson *et al.*, 1992; Landis *et al.*, 1993). However, signal transduction events have been shown to result from both ligand and antibody binding to integrins (Hynes, 1992), and the activating antibodies may be affecting more than integrin structure.

A new class of small molecule anti-inflammatory drugs, the leumedins, has been shown to prevent the adherence of neutrophils to keyhole limpet hemocyanin and to endothelial cells (Bator *et al.*, 1992). The leumedin NPC 15669 also was shown to reduce the fMLP-induced upregulation of Mac-1 on neutrophils (Bator *et al.*, 1992). *In vivo*, NPC 15669 has been shown to inhibit neutrophil recruitment and reduce tissue damage in rats with acetic acid-induced colitis (Noronha-Blob *et al.*, 1993a), to reduce mortality associated with sepsis in rats (Noronha-Blob *et al.*, 1993b) and with endotoxin administration to mice (Burch *et al.*, 1993). NPC 15669 also was effective in blocking lung injury associated with cardiopulmonary bypass in pigs; this was associated with inhibition of upregulation of CD18 on neutrophils (Bator *et al.*, 1993). It was suggested that leumedins act in part by preventing the increase in cell surface Mac-1 level which results from cell

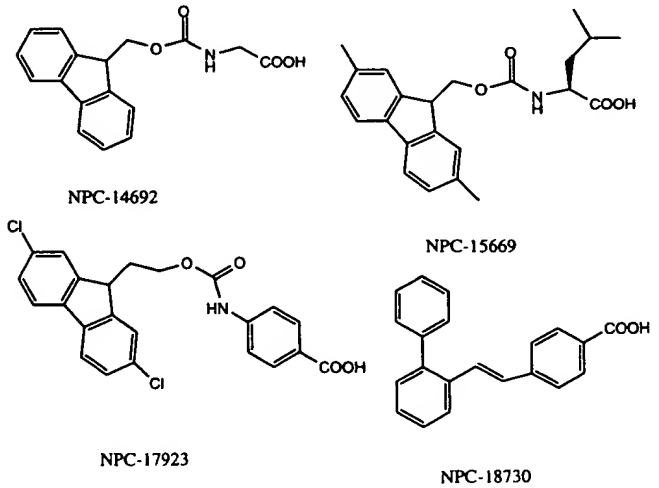

Fig. 1. Chemical structures of leumedins. NPC 14692 (N-fluorenyl-9-methoxycarbonyl-glycine), NPC 15669 (N-[9H-(2,7-dimethylfluorenyl-9-methoxy)-carbonyl]-L-leucine), NPC 17923 (N-[9H-(2,7-dichlorofluorenyl-9-ethoxy)carbonyl]-4-aminobenzoic acid; Perumattam *et al.*, 1994) and NPC 18730 (4-(2-biphenyl)-E-ethenyl benzoic acid).

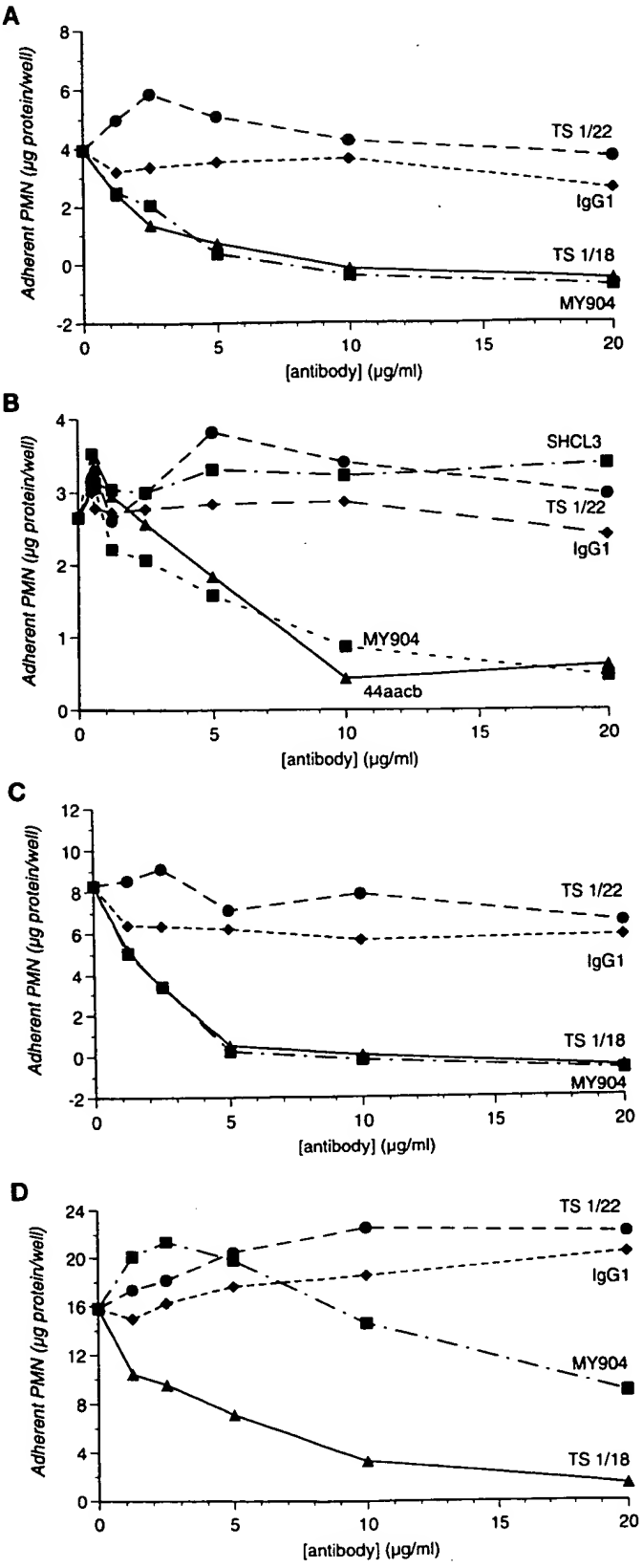

Fig. 2. Adherence of neutrophils to serum is Mac-1-dependent. PMN were mixed with antibodies as indicated and preincubated in serum-coated wells in duplicate for 15 min at 37°C. Adherence was quantitated after treatment with: A, 100 nM fMLP for 10 min; B, 1 µM PAF for 10 min; C, 0.1 µM C5a for 20 min; and D, 0.6 nM TNF-α for 20 min at 37°C.

TABLE 1

Inhibition of neutrophil adherence by leumedins

PMN were mixed with leumedins (7 dilutions in the appropriate range) and added to serum-coated wells in duplicate. After incubation for 10 min at 37°C, adherence was induced as stated in the legend to figure 1.

	fMLP	C5a	PAF	TNF-α
	Mean IC_{50} ± S.E.M.			
	μM (n)			
NPC 14692	564 ± 150 (4)	493 ± 124 (3)	311 ± 47.2 (4)	282 ± 17.0 (2)
NPC 15669	6.03 ± 3.92 (4)	92.4 ± 45.4 (3)	31.7 ± 7.8 (5)	41.7 ± 7.4 (9)
NPC 17923	45.4 ± 8.3 (4)	26.3 ± 7.2 (3)	17.8 ± 10.4 (5)	9.80 ± 1.43 (6)
NPC 18730	15.4 (2)	6.41 ± 3.37 (3)	2.62 ± 0.76 (9)	3.15 ± 0.45 (6)

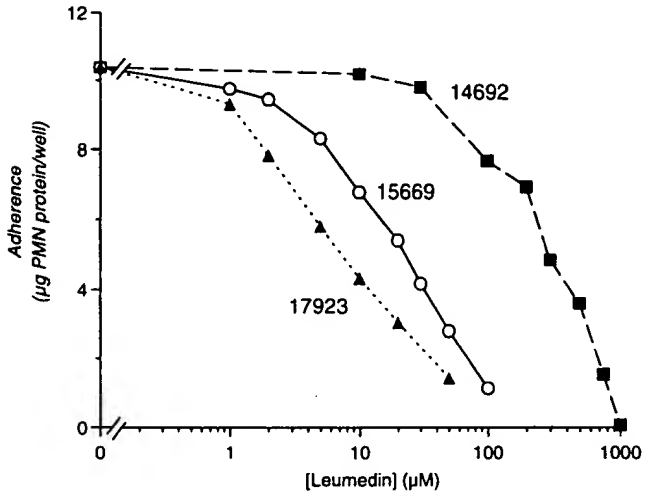

Fig. 3. Effect of leumedins on adherence of TNF-α-stimulated PMN. PMN were incubated with leumedins for 10 min at 37°C. Adherence was quantitated after activation with 0.6 nM TNF-α for 20 min at 37°C.

activation. We demonstrate here that leumedins inhibit neutrophil adherence *in vitro* independent of any effects on Mac-1 level. We also demonstrate that leumedins do not prevent the appearance of the CBRM1/5 epitope on Mac-1 after neutrophil activation. This suggests that these two changes in Mac-1 are not sufficient to bring about the adherence of neutrophils.

Materials and Methods

Experimental Design

Isolation of PMN. Blood was drawn from human volunteers (free of any medication for at least 48 hr) into syringes containing heparin (1 U/ml). Blood (30 ml) was layered over a gradient of 10 ml of 1.077 g/ml of Histopaque and 15 ml of 1.119 g/ml of Histopaque (Sigma Chemical Co., St. Louis, MO) and centrifuged at room temperature, 400 × *g* for 20 min (English and Andersen, 1974). All subsequent manipulations were carried out at room temperature, with endotoxin-free reagents and using polypropylene pipettes and tubes, to prevent activation of the PMN. The pinkish PMN layer immediately above the RBC was collected and washed 2 × by centrifugation at 400 × *g* for 10 min with HBSS. Pellets were suspended in 8 ml of 0.9% NaCl and RBC were lysed by the addition of 24 ml of water for 40 sec, followed by the addition of 8 ml of 3.6% NaCl. After centrifugation at 200 × *g* for 8 min, RBC ghosts were aspirated from the top

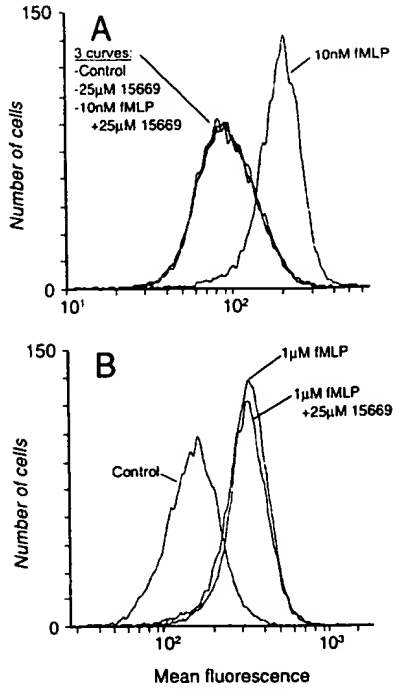

Fig. 4. Effect of NPC 15669 on fMLP induction of Mac-1 upregulation. Neutrophils were preincubated for 10 min at 37°C with or without 25 μM 15669. fMLP was added to the samples as indicated, at 10 nM (A) or 1 μM (B), and incubation continued for 20 min. Cells were cooled on ice/water and total Mac-1 level was quantitated by staining with D12 and FACS analysis.

of the pellet, and cells were washed with HBSS. Neutrophils were identified morphologically after staining with Wrights and Giemsa stains (Junqueira *et al.*, 1986).

PMN adherence to protein substrates. Ninety-six well tissue culture plates were coated by incubation with 10% pooled human serum (North American Biologicals, Miami, FL) in PBS for 1 hr at 37°C, followed by washing 4 times with PBS. Leumedins were prepared in dimethylsulfoxide as 100 to 1000 × stocks. Unless otherwise indicated, duplicate 0.1-ml aliquots of PMN (4×10^6/ml) were incubated with leumedins or antibodies in serum-coated wells for 10 min at 37°C. Activators were added and adherence was carried out for 10 to 20 min as indicated. Nonadherent cells were aspirated and wells were washed 2 times with warm PBS with calcium and magnesium; with blotting of the inverted plate after each aspiration. Adherent PMN were quantitated using a BCA protein assay (Pierce Chemicals, Rockford, IL). Absorbance was measured using a Bio-Mek plate reader, zeroed on serum-coated wells to which no PMN were added, which contained less than 1 μg of protein per well. Duplicates typically differed by less than 10%. Adherence of unstimulated PMN was always less than 20% (typically less than 10%) that of stimulated PMN. Ten micrograms of adherent PMN represents ~25% of the 4×10^5 cells added per well. Levels of adherent PMN were variable, depending on the PMN donor and the activator used. TNF-α resulted in a reproducably high level of adherence, whereas the response to fMLP was donor-dependent. All results are representative of at least two experiments.

Flow cytometric analysis of neutrophil adhesion molecule expression. PMN were prepared as above, but without lysis of the RBC. (In some preparations, after centrifugation on Histopaque, the PMN were put on ice and the washes were carried out at 4°C. Cells were slowly warmed at room temperature for 10 min.) PMN (3×10^7/ml) were preincubated with leumedins for 10 min at 37°C or as indicated. Aliquots (50 μl) were added to v-bottomed polypropylene 96-well plates (Nunc, V.W.R., Brisbane, CA), and activators were added for the indicated time. Plates were cooled on ice/water and all

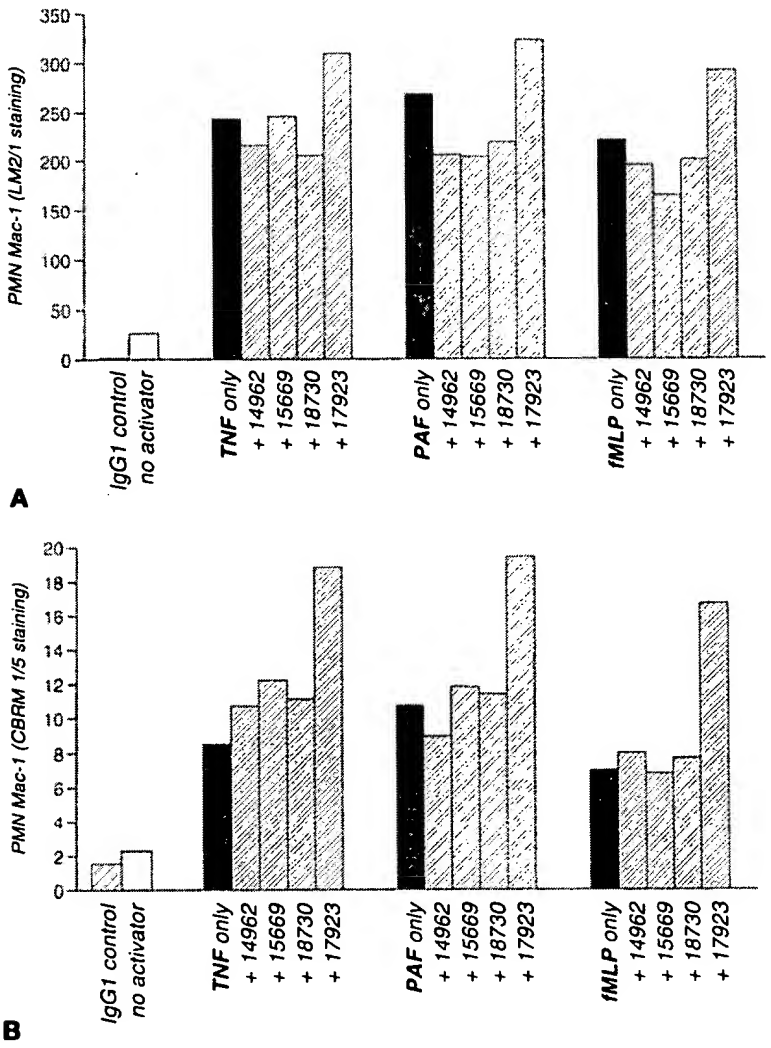

Fig. 5. Effect of leumedins on activation-induced changes in Mac-1 level and CBRM1/5 expression. Neutrophils were preincubated with leumedins [NPC 14692 and NPC 15669, 100 μM; NPC 18730, 75 μM; and NPC 17923, 20 μM] at 37°C for 10 min, followed by addition of activator and incubation at 37°C as follows: 0.6 nM TNF-α for 20 min and 1 μM PAF or 100 nM fMLP for 10 min. Cells were then cooled on ice/water and stained with IgG1 (controls), LM2/1 to quantitate total Mac-1 level (A) or CBRM1/5 (B), followed by fluorescein-goat-anti-mouse IgG. Antibody binding to cells was quantitated by FACS analysis.

subsequent steps were carried out on ice. Antibodies (in 10% heat-inactivated FBS in PBS + 10 mM sodium azide) were added to 20 μg/ml. After incubation for 1 hr, cells were washed 2 × with PBS-FBS-azide, then were incubated for 30 min with 20 μg/ml of goat antimouse IgG labeled with fluorescein (Pierce Chemicals) in PBS-FBS-azide. PMN were washed with PBS + 10 mM azide, RBC were lysed with cold FACS lyse (Becton Dickinson, San Jose CA) and cells were suspended in 0.5% formaldehyde in FACS sheath fluid (Haema-Line 2, Becton Dickinson) overnight before flow cytometry (FACS-can, Becton Dickinson). Neutrophils were gated from contaminating cell types and debris by using forward and side scatter measurements. Data are expressed as the mean fluorescence of the neutrophil population. In some experiments, RBC lysis was carried out before the use of PMN, resulting in a partial activation of the cells. Whereas the basal level of Mac-1 was increased by lysis of the RBC, this did not significantly affect the experimental results. All results are representative of at least two experiments.

Materials. The anti-Mac-1 antibody, D12, and the CD11c/CD18 antibody, SHCL3 (Stacker and Springer, 1991), were obtained from Becton Dickinson, and control IgG1 (MOPC21) was obtained from Sigma Chemical Co. CBRM1/5 was provided by Timothy Springer (Diamond and Springer, 1993). Hybridomas producing the anti-Mac-1 antibody, LM2/1 (Miller *et al.*, 1986), the LFA-1 blocking antibody, TS1/22, the CD18 blocking antibody, TS1/18 (Sanchez-Madrid *et al.*, 1983), and the Mac-1 blocking antibodies, MY904 and 44aacb (Dana *et al.*, 1986), were obtained from American Type Culture Collection (Rockville, MD). All antibodies were purified before use; an exception was the use of hybridoma supernatant containing CBRM1/5 in figure 5. Human recombinant TNF-α was from R & D Systems (Minneapolis, MN); human recombinant C5a, bovine heart PAF and fMLP were from Sigma Chemical Co. Structures of the leumedins, confirmed by NMR spectra and elemental analysis, are shown in figure 1.

Results

Mac-1 mediates adherence of PMN to protein substrates. The adherence of PMN to endothelial cells or keyhole limpet hemocyanin has been shown previously to be inhibited by the leumedin NPC 15669, with IC_{50} values from 14 to 48 μM (Bator *et al.*, 1992). Both selectins and β_2 integrins can contribute to neutrophil adherence to endothelial cells (Smith *et al.*, 1991). In order to assess the effects of leumedins on Mac-1 alone, we used human serum as a PMN adhesion substrate. PMN have been shown to bind serum, fibrinogen, keyhole limpet hemocyanin and denatured albumin *via* Mac-1 (Altieri *et al.*, 1988; Hughes *et al.*, 1992; Davis, 1992). We measured the adherence of activated PMN to serum-coated tissue culture plastic and used blocking antibodies to verify the contributions of β_2 integrins to this process. As shown in figure 2, antibodies to CD11b (MY904 or 44aacb), or CD18 (TS1/18), completely block the adherence of PMN activated with fMLP, PAF or C5a to serum-coated wells. However, whereas an anti-CD18 antibody blocked the

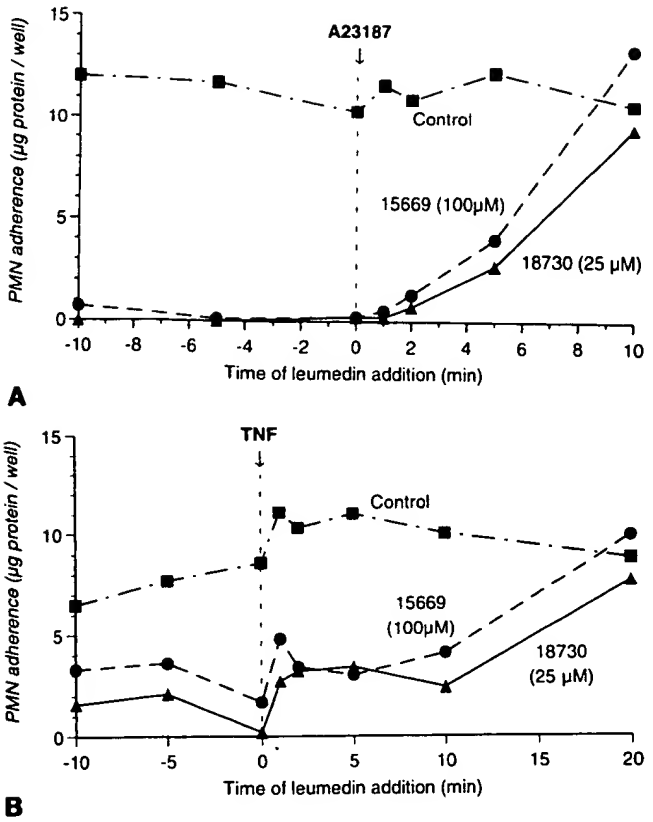

Fig. 6. Timing of leumedin effect relative to neutrophil activation. PMN were aliquotted into serum-coated wells and incubated at 37°C. A23187 (2.5 μM; A) or 0.6 nM TNF-α (B) were added at time 0. NPC 15669 (100 μM), 25 μM NPC 18730 or dimethylsulfoxide (control) were added at time = −10, −5, 0, 1, 2, 5 or 10 min as indicated. Adherence was quantitated at 10 min after A23187 (A) or 20 min after TNF-α (B).

adherence of TNF-α-activated PMN to serum, inhibition by anti-CD11b antibodies was not complete, ranging from 50 to 80% in several experiments. Blocking antibodies to CD11a (TS1/22) and CD11c (SHCL3) did not inhibit PMN adherence to serum under any conditions. Thus, PMN adherence to serum is mediated primarily by CD11b/CD18, with some contribution of other β_2 integrins to TNF-α-activated PMN.

Leumedins block Mac-1-mediated adherence of PMN to serum. The adherence of PMN activated with fMLP, C5a, PAF or TNF-α to serum is blocked by leumedins as shown in table 1. Inhibition by leumedins is dose-dependent and complete, as shown in figures 3, 6 and 7. For NPC 14692, 17923 and 18730, IC_{50} values are lowest for PAF and TNF activation, and highest for fMLP activation. NPC 15669 is unusual in that it is more potent against fMLP-treated PMN compared to other activators. Further investigation showed this anomaly to be due to the ability of NPC 15669 to inhibit the binding of fMLP to its cell surface receptor. The binding of [^{3}H]fMLP to neutrophils was blocked with an apparent IC_{50} of ~3 μM NPC 15669 (J. M. Jones, unpublished observations) (Smith *et al.*, 1995).

Leumedins do not block upregulation of neutrophil surface Mac-1 *in vitro*. It has been shown that NPC 15669 can reduce the upregulation of Mac-1 on neutrophils induced by 10 nM fMLP *in vitro* (Bator *et al.*, 1992). Because of the possibility that this effect was due to blocking of the fMLP receptor by NPC 15669, we repeated this experiment and varied the level of fMLP. As expected, 100 μM NPC 15669 inhibited Mac-1 upregulation induced by 10 nM fMLP completely. However, 100 μM NPC 15669 did not inhibit Mac-1 upregulation induced by 1 μM fMLP (fig. 4). This is consistent with competition between NPC 15669 and fMLP for binding to the fMLP receptor.

We assessed the effects of leumedins on cell surface Mac-1 expression induced by activators other than fMLP. At levels which block TNF-α- or PAF-induced adherence, leumedins do not block the upregulation of Mac-1 induced by TNF, PAF or 100 nM fMLP (fig. 5A). PMN treated with NPC 17923 consistently expressed higher levels of Mac-1 than cells treated with activator alone.

Leumedins inhibit some process downstream of PMN activation. Several observations indicate that, although NPC 15669 may bind to the fMLP receptor (above), leumedins additionally target an event subsequent to activation at the level of cell surface receptors. First, leumedins block neutrophil adherence induced by the calcium ionophore A23187, which activates PMN by allowing calcium to enter the cell (fig. 6A). Furthermore, leumedins can be added to PMN several minutes after A23187, and still substantially inhibit adherence to serum (fig. 6A). PMN can be treated with TNF-α for up to 10 min in serum-coated wells, allowing complete activation of cell surface receptors by TNF-α; addition of leumedins for the following 10 min is sufficient to block adherence (fig. 6B). TNF-α is somewhat unique in that a 5- to 10-min delay is observed in PMN responses including adherence, compared to fMLP or PAF (Shimizu *et al.*, 1993; unpublished observations).

Leumedins do not block induction of the CBRM1/5 epitope on Mac-1. Activation of PMN leading to Mac-1-mediated adherence also leads to the appearance of "activation" epitopes on Mac-1, recognized by antibodies such as CBRM1/5 (Diamond and Springer, 1993). We assessed the effects of leumedins on the expression of the CBRM1/5 epitope. Figure 5B shows that TNF-α, PAF and fMLP all induce a 5-fold increase in CBRM1/5 epitope expression on PMN, and that this increase is not blocked by leumedins at levels sufficient to block PMN adherence. NPC 17923 reproducably caused the CBRM1/5 epitope to be expressed at a level above that induced by activation alone.

Leumedins block an event occurring before plating of TNF-α- or PAF-activated PMN, but after plating of A23187-activated PMN. Leumedins could target a process occurring during cellular activation in parallel to changes in Mac-1. Alternatively, leumedins could target an event resulting from the recognition of adhesion partners by Mac-1. In order to distinguish these possibilities, PMN were preincubated in tubes with activator plus/minus the indicated concentrations of leumedin (fig. 7). The incubation times necessary to achieve expression of the CBRM1/5 epitope on Mac-1 were determined and were found to be 5 min for PAF and 15 min for TNF-α (fig. 8). (The increase in total Mac-1 level, as measured by LM2/1 binding, occurred with similar kinetics, data not shown.) After a 5-min preincubation with A23187 or PAF, or 15 min with TNF-α, PMN were added to serum-coated wells containing identical concentrations of leumedin, or diluted 2-fold into medium alone. PMN were allowed to adhere a further 10 min, and the resulting adherence was graphed as a function of the leumedin level present in preincubation and/or adherence buffers. This dilution protocol was used instead of washing cells to completely remove leu-

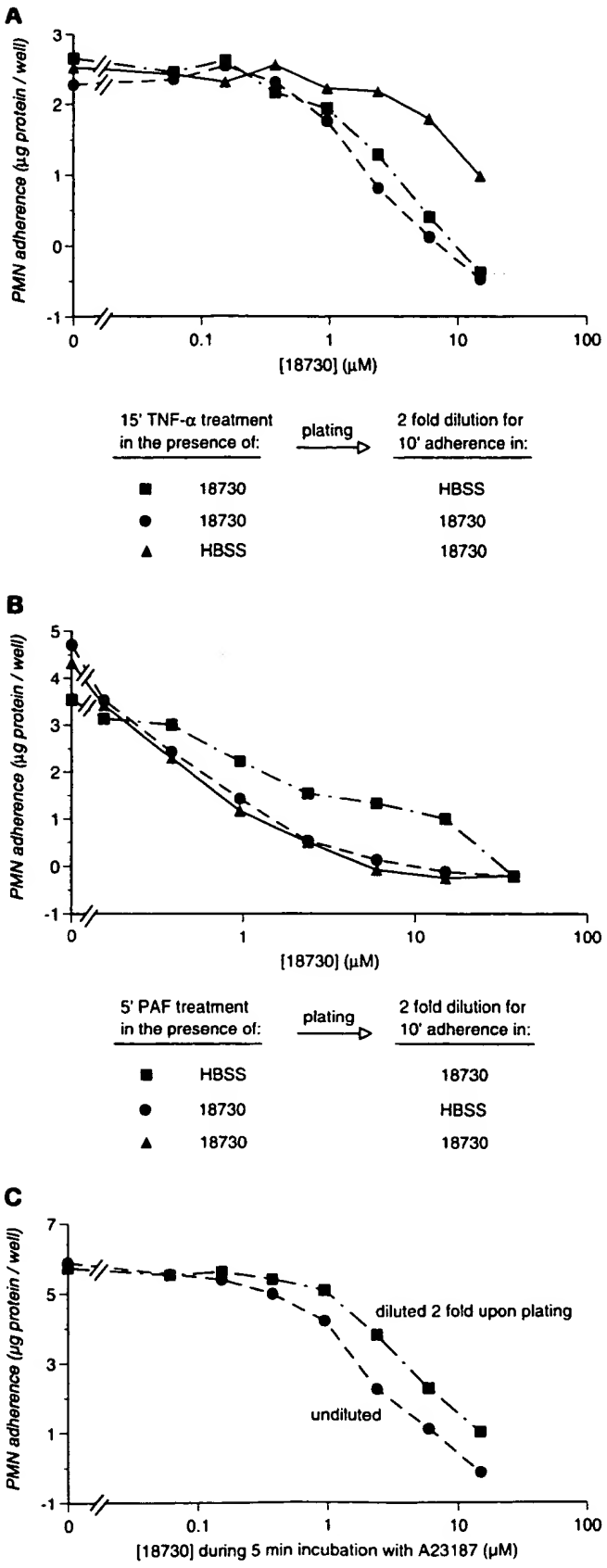

Fig. 7. Leumedins block a process occurring before plating of TNF-α- or PAF-activated, but after plating of A23187-activated, neutrophils. PMN were incubated with the indicated levels of NPC 18730 or in HBSS alone, at 37°C for 5 min. Activators were added to PMN and incubation continued in tubes to allow activation of Mac-1 as follows: A, 0.6 nM TNF-α, 15 min; B, 2 µM PAF, 5 min; and C, 2.5 µM A23187, 5 min. Cells were then plated in serum-coated wells containing an equal volume of HBSS alone, or HBSS with 18730 at the indicated concentration. Adherence was quantitated after 10 min at 37°C.

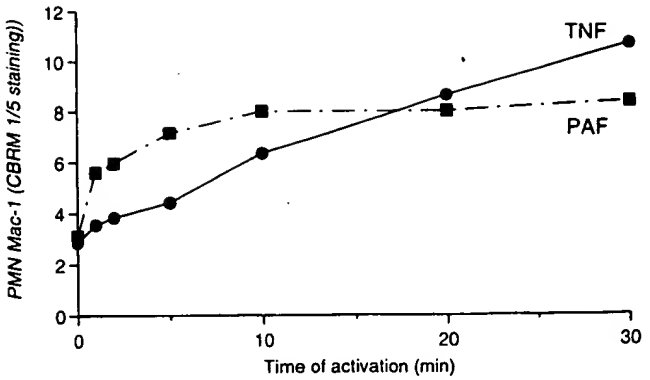

Fig. 8. Kinetic analysis of CBRM1/5 epitope induction on Mac-1. Neutrophils were warmed to 37°C, then incubated with 0.6 nM TNF-α or 2 µM PAF for the indicated times at 37°C. Cells were chilled and stained with CBRM1/5.

medin, because of the poor adherence observed after washing of activated cells. This was presumably due to missing the optimal time window for adherence after cell activation.

TNF-α and PAF differed from A23187 in the results obtained. With TNF-α and PAF (fig. 7, A and B), inhibition of adherence was dependent on the presence of NPC 18730 during the activation period. The apparent IC_{50} did not shift if NPC 18730 levels were diluted upon plating. This is confirmed by the results obtained if PMN were preincubated without NPC 18730, then added to diluent containing NPC 18730 upon plating. In this case (fig. 7, A and B), inhibition of adherence by NPC 18730 was much less effective. This means that the IC_{50} was determined during the preincubation step in the tubes, in parallel with increased Mac-1 level and CBRM1/5 epitope expression.

This was not the case for A23187; the apparent IC_{50} shifted 2-fold to the right when NPC 18730 was diluted upon plating, indicating that the leumedin concentration after plating determined the inhibitory effect (fig. 7C). In this case the process targeted by leumedins took place only after exposure to an adhesion partner. A23187, simply by increasing intracellular calcium, is not likely to mimic the full range of effects elicited by TNF-α or PAF. Complete activation may depend on ligation of integrins by their substrates.

Discussion

When neutrophils are activated with TNF-α or other factors, β_2 integrins on their surface gain the ability to bind to ICAM-1 and other counter receptors. This correlates with and may be due to conformational changes in LFA-1 and Mac-1 detected by the appearance of "activation" epitopes (Diamond and Springer, 1993, 1994; Lollo *et al.*, 1993; Elemer and Edgington, 1994). Concurrently, changes in the levels of cell surface adhesion molecules take place; Mac-1 level is increased by 10-fold (Miller *et al.*, 1987; Vedder and Harlan, 1988; Schleiffenbaum *et al.*, 1989), and L-selectin is "shed" (Kishimoto *et al.*, 1989). We have assessed the effects of the anti-inflammatory drugs, leumedins, on the levels of Mac-1 and L-selectin, as well as on the expression of an

activation epitope on Mac-1. Leumedins do not block the shedding of L-selectin from activated neutrophils (G. Endemann, C. Bryant, Y. Feng and D. Liu, manuscript in preparation). When neutrophils are treated with a variety of activating agents, including TNF-α, PAF and fMLP, leumedins do not block the resulting increase in cell surface Mac-1 level, or expression of the CBRM1/5 epitope on Mac-1. These findings suggest that changes in Mac-1 level and CBRM1/5 epitope expression are not sufficient to cause the adherence of neutrophils.

The previously reported ability of NPC 15669 to block the fMLP-induced upregulation of Mac-1 (Bator *et al.*, 1992) appears to be due to competition for binding to the fMLP receptor (see "Results") (Smith *et al.*, 1995). In pigs undergoing cardiopulmonary bypass, NPC 15669 was found to partially block the upregulation of CD18 on neutrophils attributed to the generation of C5a (Bator *et al.*, 1993). This may reflect indirect effects of NPC 15669. Others have reported that NPC 15669 can inhibit eosinophil chemotaxis without affecting Mac-1 upregulation (Kaneko *et al.*, 1994). The inability of leumedins to block the upregulation of Mac-1 on neutrophils is not inconsistent with their ability to block adherence. The adherence of neutrophils in response to a single stimulus has been shown to utilize constitutively expressed Mac-1, whereas Mac-1 which is newly recruited to the plasma membrane appears to contribute to subsequent rounds of adherence involved in migration toward a chemotactic stimulus (Hughes *et al.*, 1992). Indirect effects of NPC 15669 on the upregulation of Mac-1 may affect neutrophil migration *in vivo*.

It is not known if CBRM1/5 binding is indicative of a conformational change in Mac-1, allowing recognition of adhesion partners. Ligand-induced conformational activation has been reported for LFA-1 (Cabanas and Hogg, 1993) and for $\alpha_{IIb}\beta_3$ (Du *et al.*, 1991). Our results indicate that CBRM1/5 epitope expression is not sufficient to allow neutrophil adherence. However, although the CBRM1/5 epitope is found on only 10 to 50% of Mac-1 molecules, the antibody completely blocks Mac-1-dependent adherence (Diamond and Springer, 1993; unpublished observations). It is not known whether other changes in Mac-1, not detected by CBRM1/5, contribute to adherence. After activation of neutrophils, and formation of the CBRM1/5 epitope, further changes in Mac-1 could take place either preceding or following binding to adhesion partners. The monoclonal antibody 7A10 is reported to bind to Mac-1 after cellular activation, and the number of 7A10 binding sites on THP-1 cells is equivalent to total Mac-1 molecules (Elemer and Edgington, 1994). In addition, other parallel or subsequent processes, for example involving cytoskeletal rearrangements, shape change and spreading, may be necessary.

The mechanism by which leumedins block the adherence of neutrophils is not known. Nonspecific effects due to cytotoxicity are not likely to contribute. All compounds have been tested for toxicity to PMN by two methods: trypan blue exclusion and the uptake and hydrolysis of calcein acetoxymethyl ester (Hamilton *et al.*, 1995; data not shown), and compounds were not used at levels resulting in toxicity. Furthermore, the effects of leumedins on resting neutrophils were fully reversible, as removal of leumedin before addition of activator rendered the cells fully competent for adherence (data not shown). Inhibition of adherence does not require blocking the initial activation of neutrophils, as measured by increased Mac-1 level, expression of an activation epitope on Mac-1 and L-selectin shedding. Leumedins are not competitive inhibitors of Mac-1, as they do not block the binding of endothelial cells to purified Mac-1 (G. Endemann and Y. Feng, unpublished data). Furthermore, the results of our time course experiments show that leumedins target an event which follows the binding of activators to cell surface receptors. Leumedins could be added up to 10 min post-TNF-α and still block adherence. Additional time course experiments were designed to distinguish processes occurring in parallel to upregulation of Mac-1 and appearance of the CBRM1/5 epitope, from those occurring as a consequence of Mac-1 binding to an adhesion partner. With TNF-α and PAF, the event targeted by leumedins occurred before the addition of activated cells to wells, and therefore is not a consequence of Mac-1 binding to an adhesion partner. With A23187, the event targeted by leumedins occurred after the addition of activated cells to wells; in this case, binding of Mac-1 to an adhesion partner may be required for complete activation.

Although this work has focused on the Mac-1-dependent adherence of neutrophils to protein substrates, the effects of leumedins are not limited to this adherence mechanism. Leumedins completely block the adherence of neutrophils to human umbilical vein endothelial cells activated with interferon-γ; this adherence is mediated by both Mac-1 and LFA-1 (Hamilton *et al.*, 1995). When E-selectin is upregulated on endothelial cells by treatment with TNF-α, the IC_{50} values for blocking of neutrophil adherence by leumedins are significantly higher (K. Madden and L. Stanton, unpublished observations), and for some leumedins may be due to cytotoxicity. These data are consistent with leumedins blocking a component of neutrophil adherence common to β_2 integrins. Leumedins appear to target an event which occurs in a late stage of neutrophil activation, and is necessary for adherence.

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